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# Induction of Interleukin-6 During Human Immunodeficiency Virus Infection

By Deborah L. Birx, Robert R. Redfield, Kathleen Tencer, Arnold Fowler, Donald S. Burke, and Giovanna Tosato

**Interleukin-6 (IL-6), a multifunctional cytokine produced in monocytes, fibroblasts, and other cell types, is induced by a variety of stimuli, including bacteria, viruses, and other cytokines. When normal monocyte cultures were exposed to a monocytotropic strain of human immunodeficiency virus (HIV), HTLV-III$_{Ba\text{-}L}$, significant levels of IL-6 bioactivity were detected in the culture supernatants after 12 to 43 days of incubation, at a time when there was associated evidence of HIV production. Similarly, when normal monocyte cultures were cocultured with peripheral blood mononuclear cells from HIV-infected individuals, HIV replication in these cultures was associated with production of IL-6. In further studies, we determined that mean serum levels of IL-6 bioactivity were abnormally elevated in HIV-seropositive individuals with stage 1/2 infection (25.2 ×/÷ 1.8 U/mL) and stage 3/4 infection (46.1 ×/÷ 1.7 U/mL) when compared with normals (1.6 ×/÷ 1.2 U/mL). In contrast, mean serum IL-6 levels were not different from normal in stage 5/6 infection (2.7 ×/÷ 1.6 U/mL). A selected group of 12 HIV-seropositive individuals (stages 1, 2, and 3) who harbored HIV capable of replicating in T cells but not in monocyte cultures had a mean serum IL-6 level of 5.3 U/mL (×/÷1.5), a value significantly lower ($P < .004$) than that measured in control HIV-seropositive individuals infected with monocytropic HIV (39 ×/÷ 1.9 U/mL). In addition, serum IL-6 levels in HIV-seropositive individuals (stages 1 through 6) correlated directly with serum immunoglobulin G (IgG) levels ($R = .74$, $P < .001$). Monocytes but not T cells are capable of a high level IL-6 production in vitro, and monocyte-derived IL-6 stimulates Ig production in activated B cells. Thus, HIV-seropositive individuals who often are infected with monocytotropic HIV and often display abnormally elevated serum IgG levels may exhibit these abnormalities as a consequence of abnormally elevated IL-6 levels induced by HIV.**



INTERLEUKIN-6, (IL-6), a recently identified phosphoglycoprotein, is emerging as a multifunctional cytokine induced by a variety of stimuli. Monocytes, fibroblasts, keratinocytes, and endometrial stromal cells produce IL-6 in vitro on stimulation with different signals, such as bacteria, bacterial products, viruses, and certain cytokines.[1-6] IL-6 stimulates liver cell cultures to produce a number of "acute-phase proteins," such as C-reactive protein, fibrinogen, and α-1-antichymotrypsin[7-9]; promotes growth and immunoglobulin (Ig) secretion in B cells[2,10]; acts as an accessory signal for T cells[11,12]; and stimulates colony formation in hematopoietic progenitor cells.[13] Other properties attributed to IL-6 include antiviral activity,[14] growth of myeloma cells,[15,16] and growth inhibition of myeloleukemic and breast carcinoma cells.[17]

The multiplicity of functions displayed by IL-6 in vitro suggests that this cytokine may also display multiple complex roles in vivo. So far, a number of studies support the view that IL-6 is a critical mediator of acute phase responses in vivo. Serum levels of IL-6 increase substantially in severe burn victims,[18] in septic individuals,[19] and during acute rejection of kidney transplants.[20] The levels of IL-6 then rapidly return to normal after resolution of the acute episode. IL-6 may also have a role in certain chronic or recurrent inflammatory states because levels of IL-6 are elevated in the serum and synovial fluid of a proportion of patients with rheumatoid arthritis.[21-24]

It was recently reported that increased messenger IL-6 levels and increased secretion of IL-6 bioactivity are induced early after exposure of normal mononuclear cells to either live or inactivated HTLV-IIIb.[25] In addition, it is known that human immunodeficiency virus (HIV)-infected individuals have abnormally elevated serum Ig levels,[26] and that a proportion of HIV-infected individuals also display abnormally elevated numbers of Epstein-Barr virus (EBV)-infected B cells in the circulation.[27] Studies of IL-6 bioactivity in vitro have shown that IL-6 stimulates the proliferation of EBV-infected B cells[2] and promotes Ig secretion in activated B cells.[8] For these reasons, we have further studied the role of HIV as an inducer of IL-6. In particular, we have examined whether HIV, isolated from infected individuals, induces IL-6 secretion in vitro. In addition, we have tested whether HIV-infected individuals have abnormally elevated serum IL-6 levels.

## MATERIALS AND METHODS

*Patient selection and serum samples.* Serum samples and mononuclear cells were obtained with informed consent from individuals at Walter Reed Army Medical Center (WRAMC) during clinical HIV staging evaluations. HIV-infected patients were staged according to the WRAMC staging criteria for HIV infection.[28] Briefly, stage 1 individuals have greater than 400 CD4 cells/mm$^3$, while stage 2 individuals have greater than 400 CD4 cells/mm$^3$ and chronic (>3 months) adenopathy. Stage 3 and 4 patients have less than 400 CD4 cells/mm$^3$ with stage 4 patients demonstrating early defects in delayed-type sensitivity. Stage 5 and 6 patients have less than 400 CD4 cells/mm$^3$ and increasing degrees of clinical immunodeficiency, with stage 5 patients having anergy or symptomatic,

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biopsy-proven thrush, and stage 6 patients demonstrating opportunistic infections generally associated with HIV disease. Kaposi's sarcoma is not considered a stage identifier and none of the patients in this study had Kaposi's sarcoma. All patients were seropositive for HIV by enzyme-linked immunosorbent assay (ELISA) and Western blot. None of the patients were acutely ill with an intercurrent illness at the time of this study. Normal sera and mononuclear cells were also obtained from healthy HIV seronegative controls.

*Virus isolation from peripheral blood.* Patient or control peripheral blood mononuclear cells (PBMCs) were cocultured both with normal monocytes and normal PBMCs, essentially as described.[29] Normal monocytes, obtained by elutriation of PBMCs (>95% nonspecific esterase positive cells[30]) were first incubated for 4 days ($5 \times 10^6$ cells/mL in 24-well tissue culture plates, 0.5 mL/well) in monocyte isolation culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 5% human sera and 1,000 U/mL colony-stimulating factor (CSF) (Cetus Corp, Emeryville, CA). Patient or control PBMCs ($2 \times 10^6$ cells in 0.5 mL isolation culture medium) were then added to the 24-well plates containing 4-day precultured normal monocytes, and incubation continued.

Normal PBMCs ($1 \times 10^6$ cells/mL in 75-cm$^2$ tissue culture flasks, 40 mL/flask) were first incubated for 3 days in culture medium (RPMI 1640 with 15% fetal calf serum (FCS), 20 mmol/L L-glutamine, all from GIBCO, and 5 μg/mL gentamicin (Sigma Chemical Co, St Louis, MO) supplemented with phytohemagglutinin (PHA) (Sigma; 1 μg/mL). During the final 60 minutes of incubation, cultures were supplemented with polybrene (Sigma; 2 μg/mL). After incubation, the PHA-stimulated and polybrene-treated PBMCs were washed, suspended ($2 \times 10^6$ cells/mL) in T-cell isolation culture medium consisting of RPMI 1640 with 15% FCS, 20 mmol/L L-glutamine, 5 μg/mL gentamicin, and 100 U/mL recombinant IL-2 (Cetus), and transferred to tissue culture tubes (Falcon 3033; 1 mL/tube; Division at Becton Dickinson, Lincoln Park, NJ). Patient or control PBMCs ($3 \times 10^6$ cells in 1.5 mL T-cell isolation culture medium) were then added to the culture tubes containing preactivated normal PBMCs, and incubation continued. All cultures were fed twice weekly by replacing 50% of the appropriate monocyte or T-cell isolation culture medium; the cell-free culture supernatants were stored at −70°C, and analyzed for the HIV-associated p24 antigen and IL-6 bioactivity. A culture was considered positive for HIV when p24 levels were ≥250 pg/mL in culture supernatants obtained in 2 consecutive weeks, as measured in a standard ELISA, using the Coulter p24 kit (Coulter Electronics, Hialeah, FL). Cultures were maintained until evidence of HIV production was obtained or until day 84. Previous results had demonstrated that incubation beyond day 84 did not improve HIV isolation.

*Monocyte infection with HTLV-III$_{Ba\text{-}L}$.* A monocytotropic strain of HIV, HTLV-III$_{Ba\text{-}L}$ (a gift of Drs S. Gartner and M. Popovic, National Cancer Institute, Bethesda, MD) was propagated and prepared as previously described.[31] Monocytes (>95% nonspecific esterase positive cells) obtained from PBMCs by elutriation,[30] were precultured for 2 days ($1 \times 10^6$ cells in 2 mL culture medium consisting of RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, and 5 μg/mL gentamicin) in 24-well plates (Costar). Triplicate cultures were then exposed to either medium or HTLV-III$_{Ba\text{-}L}$ (80,000 cpm/mL reverse transcriptase activity) or heat-inactivated (1 hour at 56°C) HTLV-III$_{Ba\text{-}L}$. After 2 days of incubation, all monocyte cultures were washed (to remove excess virus where added) and then cultured in complete culture medium. Cells were fed every 4 to 5 days by replacing 50% of the medium. Culture supernatants, obtained every 4 to 5 days, were stored at −70°C and analyzed for p24 antigen and IL-6 bioactivity.

*Assay for IL-6 activity.* B9 cells were used in a standard assay for IL-6 bioactivity.[32] Serial 1 to 4 dilutions of culture supernatants, sera, and recombinant *Escherichia coli*-derived IL-6 (Genzyme, Boston, MA; used as a standard throughout) were distributed in triplicate to 96-well round bottom plates. Exponentially growing B9 cells ($2 \times 10^3$ cells/well) that had been extensively washed free of IL-6 were added to the plates. Culture medium (0.2 mL/well) consisted of RPMI 1640 supplemented with 10% FCS and $10^{-5}$ mol/L 2-mercaptoethanol. Plates were incubated for 84 hours at 37°C in a humidified (5% carbon dioxide) incubator, then pulsed with 0.5 μCi/well [$^3$H]-thymidine (6.7 Ci mmol; New England Nuclear) for the last 4 hours of culture, harvested with an automated cell harvester, and counted (LKB beta plate system). Arithmetic means of triplicate wells were calculated and used for analysis. Activity is expressed as IL-6 U where 1 U is defined as causing one-half maximal B9 cell proliferation under the conditions described. An IL-6 concentration of 1 U/mL corresponds to approximately 7 pg/mL of a recombinant IL-6 laboratory standard, and to 5.5 U/mL of the interim IL-6 National Institutes of Health reference standard (prep 88/514).

*Soluble IL-2 receptor determination.* Serum samples were analyzed for content of soluble IL-2 receptor (sIL-2R) using a standard ELISA (T Cell Sciences[33]).

*Radiolabeling, immunoprecipitation, and polyacrylamide gel electrophoresis (PAGE).* HIV positive (p24 > 250 pg/mL) cultures of normal monocytes infected with HIV-III$_{Ba\text{-}L}$, established as described above, were washed three times with methionine deficient MEM (GIBCO), then incubated in the same medium (2 mL) supplemented with 1% methionine-deficient FCS, 2 mmol/L L-glutamine, and 100 μCi/mL [$^{35}$S]-methionine (New England Nuclear; 100 μCi/mmol) for 48 hours. After incubation, supernatants of triplicate cultures (6 mL) were precipitated at 4°C with a saturated solution of ammonium sulfate (50% vol/vol), centrifuged at 4,000g for 30 minutes, and the resulting pellets immunoprecipitated, as previously described.[30] A rabbit heterologous antiserum to *E coli*-derived IL-6[1] and a control normal rabbit serum were used as immunoprecipitating agents at a dilution of 1:200. Immunoprecipitated material was electrophoresed through a 12.5% PAGE containing 0.1% sodium dodecyl sulfate (SDS).[34] After electrophoresis, the gels were fixed, treated with Enlightening (New England Nuclear), dried, and autoradiographed at −70°C.

*Statistical analysis.* Geometric means, standard error, standard deviations of the means, Student's *t*-test, nonparametric Spearman Rank correlations, and correlation coefficients were calculated using conventional formulas.

## RESULTS

***HIV replication in normal monocytes is associated with IL-6 secretion.*** It was previously reported that normal monocytes exposed to the laboratory strain of HIV, HTLV-IIIb, display a transient increase in IL-6 messenger RNA after 2 hours and produce increased levels of IL-6 bioactivity after 24 hours.[25] Both events were observed with either live or inactivated HIV, demonstrating that monocyte infection was not required. We have now examined whether HIV-replication in normal monocyte cultures is associated with IL-6 secretion. To this end, normal peripheral blood monocyte-enriched populations were infected with the monocytotropic strain of HIV, HTLV-III$_{Ba\text{-}L}$, and cultures were tested for HIV and IL-6 production. As expected, in each of three experiments monocytes exposed to HTLV-III$_{Ba\text{-}L}$ had evidence of viral production beginning on day 7, as demon-

strated by the detection of greater than 250 pg/mL of p24 antigen (not shown). Also, monocyte cultures exposed to either medium alone or heat-inactivated HTLV-$III_{Ba\text{-}L}$ had no evidence of viral production (p24 < 10 pg/mL). As shown in Fig 1, in each of these experiments monocytes exposed to HTLV-$III_{Ba\text{-}L}$ secreted IL-6 bioactivity in the supernatant beginning on day 11, and increasing thereafter. No IL-6 bioactivity above background (medium alone) was detected in culture supernatants when the monocytes were exposed to medium alone (not shown) or medium supplemented with heat-inactivated virus (representative results from one of three experiments are shown).

To ensure that IL-6 was produced in these mononuclear cell cultures infected with HIV, [$^{35}$S]-labeled supernatants were immunoprecipitated with a rabbit heteroantiserum to *E coli*-derived IL-6 and the immunoprecipitates analyzed by SDS-PAGE under reducing conditions. As shown in Fig 2, the characteristic bands attributable to IL-6[2,10] could be visualized in HIV-infected culture supernatants immunoprecipitated with an anti–IL-6 but not a control serum. Thus, monocyte cultures productively infected with HTLV-$III_{Ba\text{-}L}$ secrete IL-6 in the supernatants.

*Natural isolates of HIV induce IL-6 secretion in human mononuclear cells.* In further studies, we have examined whether natural isolates of HIV from infected individuals might also induce IL-6 production in culture. To this end, highly purified monocyte preparations (>95% nonspecific esterase positive cells) were cocultured with either normal mononuclear cells or mononuclear cells from HIV-infected individuals. At weekly intervals for 8 weeks, beginning on day 8, culture supernatants were analyzed for evidence of HIV infection. As expected, normal monocytes cocultured with normal mononuclear cells had no evidence of HIV production at any time point, as determined by measure of

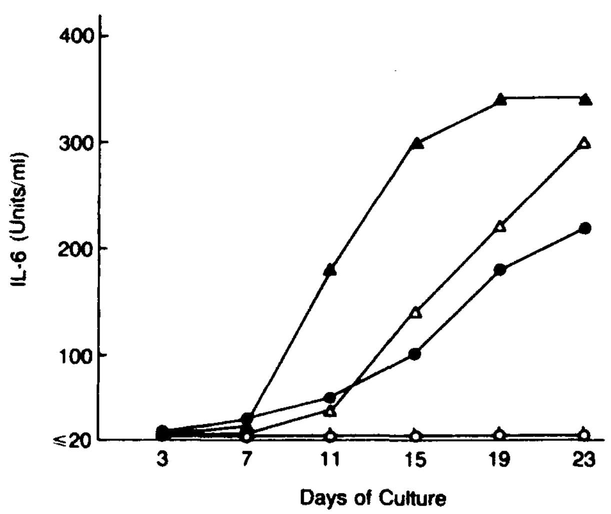


Fig 1. HTLV-$III_{Ba\text{-}L}$ stimulates IL-6 secretion in monocyte cultures. Supernatants of peripheral blood monocytes, exposed to either live (●—●, △—△, ▲—▲) or heat-inactivated (○—○) HIV were tested for IL-6 bioactivity at the indicated time points. One unit of IL-6 bioactivity is defined as the activity inducing one-half maximal proliferation of the target B9 cells.

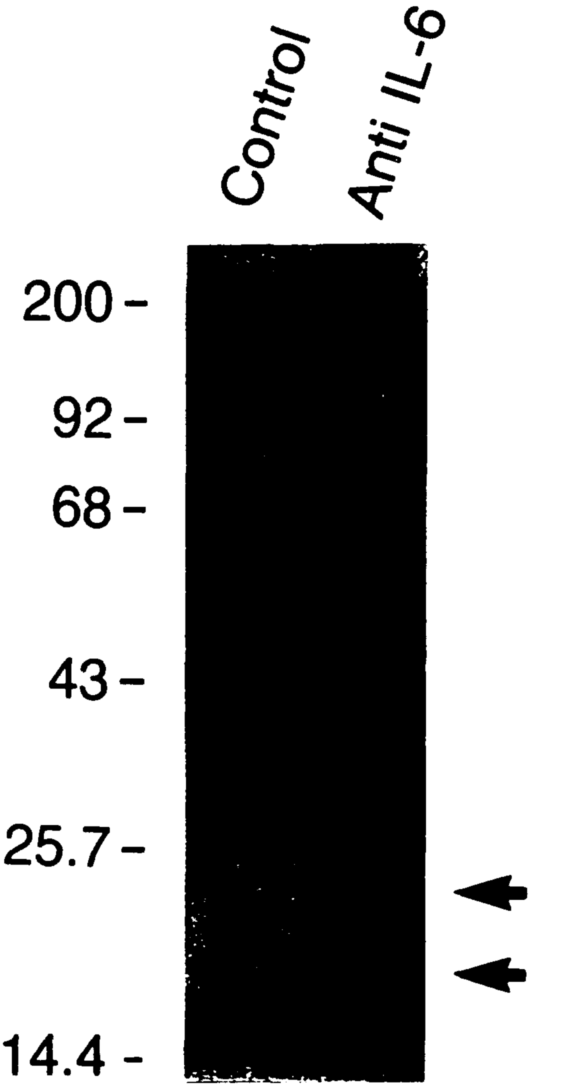


Fig 2. Detection of IL-6 protein in HIV-stimulated monocyte culture supernatants. $^{35}$S-labeled supernatants of monocytes exposed to HTLV-$III_{Ba\text{-}L}$ and producing HIV (p24 levels in the supernatant >250 pg/mL) were immunoprecipitated with either a control normal rabbit serum or an antihuman IL-6 serum (both at a dilution of 1:200). Immunoprecipitates were analyzed by electrophoresis under reducing conditions and autoradiographed.

the HIV-associated antigen, p24, in the culture supernatants. In contrast, using the same assay system, monocyte cultures incubated with mononuclear cells from HIV-seropositive individuals had evidence of HIV production in 12 of 24 samples tested. When monocyte supernatants were tested for IL-6 bioactivity in a standard growth assay of B9 cells, we found that the levels of IL-6 bioactivity were significantly higher in monocyte supernatants with elevated p24 levels when compared to monocyte supernatants with low p24 levels ($P < .04$ Spearman Rank test). Table 1 shows the results of p24 antigen and IL-6 bioactivity determinations in monocyte culture supernatants obtained on day 84 of incubation or at an earlier time point (geometric mean = 36 days)

**Table 1. IL-6 Secretion in Normal Monocyte Cocultured With Mononuclear Cells From Normal or HIV-Seropositive Individuals**

| Cocultures | No. | p24 Levels (pg/mL) | IL-6 Activity (U/mL) Mean x/÷ SEM |
|---|---|---|---|
| Normal (HIV-seronegative) | 5 | <10 | 6.7 x/÷ 5 |
| HIV seropositive | 12 | <10 | 5.1 x/÷ 2.5 |
| HIV seropositive | 12 | >250 | 48 x/÷ 6.3 |

Cell-free culture supernatants of monocyte-enriched populations cocultured with either normal or HIV-seropositive PBMC were tested in parallel for the presence of the HIV-associated antigen p24 and for IL-6 bioactivity. p24 Levels were determined by ELISA; a culture was considered HIV-positive if p24 levels in the supernatants were greater than 250 pg/mL in 2 consecutive weeks. IL-6 levels (expressed in units per milliliter) were determined by a standard growth assay for B9 cells. Data shown reflect parallel determinations on day 84 of coculture or at an earlier time point for HIV-positive cultures (mean = 36 days). Culture medium used for virus isolation had a mean of 2 x/÷ 1.3 U/mL of IL-6.

when p24 levels were found to be greater than 250 pg/mL in 2 consecutive weeks. These findings strongly suggest that HIV, isolated from the blood of HIV-seropositive individuals, induces secretion of IL-6 bioactivity in mononuclear cell cultures.

*Serum IL-6 levels are elevated in HIV-seropositive individuals.* Because HIV, isolated from infected individuals, induced IL-6 production in mononuclear cell cultures, we asked whether HIV-infected individuals might have abnormally elevated serum IL-6 levels. To this end, serum samples from 10 normal adults and 40 individuals with HIV infection were tested for the presence of IL-6 as determined by the B9 cell growth assay. As shown in Fig 3, the mean content of IL-6 in normal serum was 1.6 x/÷ 1.2 U/mL (geometric mean x/÷ SEM). In contrast, the mean serum content of IL-6 in 15 HIV seropositive individuals with stage (1/2) disease was 25.2 x/÷ 1.8 U/mL, and in HIV-seropositive individuals with stage (3/4) disease 46.0 x/÷ 1.7 U/mL. Both mean serum IL-6 values are significantly different from normal ($P < .001$). HIV-seropositive individuals with late stage (5/6) disease were found to have a mean serum IL-6 content of 2.7 x/÷ 1.6 U/mL, a value not different from normals ($P = 1.0$).

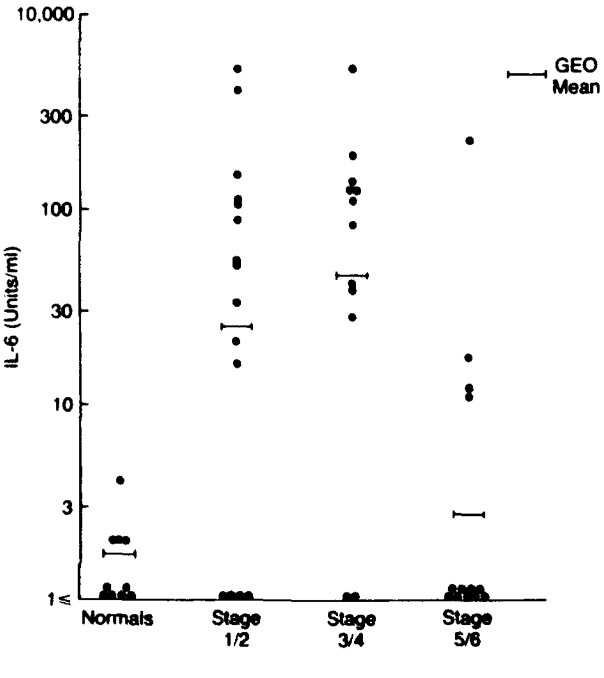


**Fig 3.** IL-6 bioactivity content in normal and HIV-seropositive sera. Serially diluted sera were tested for IL-6 bioactivity in a standard assay for IL-6, as described in Materials and Methods. One unit of IL-6 bioactivity is defined as the activity inducing one-half maximal proliferation of the target B9 cells.

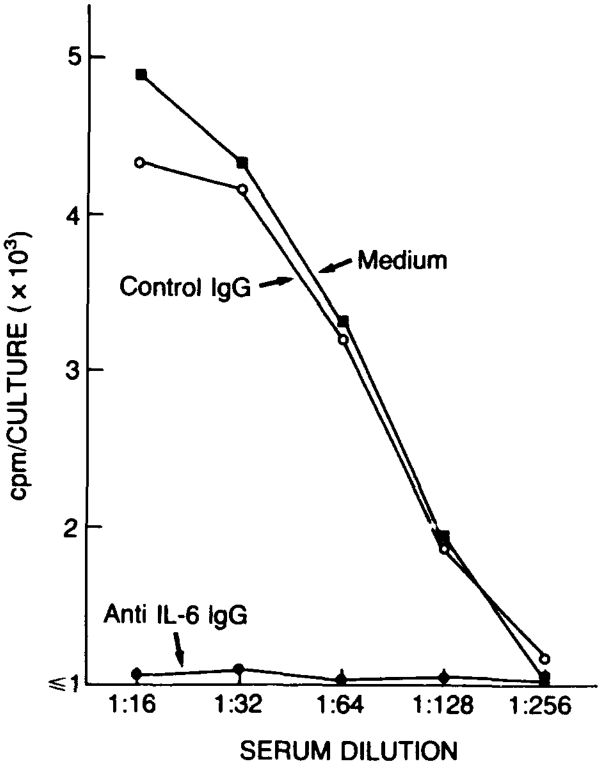


**Fig 4.** Neutralization of serum IL-6 bioactivity by an antibody to human IL-6. An appropriately diluted (1:16 to 1:256) HIV-seropositive serum was preincubated for 1 hour with either medium, or control rabbit IgG (20 μg/mL) or rabbit IgG anti-human IL-6 (20 μg/mL). After incubation, B9 cells ($3 \times 10^3$ cells/well) were added to the wells, and culture continued for 48 hours. Proliferation was measured by [$^3$H]-thymidine incorporation during the final 4.5 hours of the culture. Results are expressed as cpm/culture.

B9 cells have been reported to selectively respond to IL-6 and not to proliferate in response to other known growth factors.[28] However, to ensure that IL-6 in the serum was responsible for growth stimulation of the IL-6–dependent B9 cells, neutralization experiments were performed as described.[2] Medium, control rabbit IgG (anti-human IL-1$\beta$, 20 μg/mL,[31] or rabbit IgG anti-human IL-6 (20 μg/mL[1]) were preincubated for 1 hour at 37°C in microtiter plates with either medium or appropriately diluted HIV seropositive sera (culture volume 0.1 mL). After incubation, B9 cells ($2 \times 10^3$ cells in 0.1 mL) were added to the wells, and culture continued for 3 days. As shown in a representative experiment (Fig 4), a rabbit antibody to highly purified recombinant human IL-6, but not a control antibody, neutralized growth stimulation of B9 cells induced by serum from an

HIV-seropositive individual. These findings strongly suggest that HIV-positive individuals (stages 1 through 4) generally have abnormally high levels of IL-6 in their serum.

*Serum levels of IL-6 directly correlate with serum levels of IgG, but not of soluble IL-2 receptor (sIL-2R).* In vitro studies have demonstrated that IL-6 promotes B-cell proliferation[2,30] and Ig production.[10] HIV-infected individuals often have elevated serum Ig, particularly of the IgG isotype.[26] We have now examined whether serum levels of IL-6 in HIV seropositive individuals (stages 1 through 6) might correlate directly with serum levels of IgG in the same individuals. As shown in Fig 5, in 40 HIV seropositive individuals there was a direct relationship between serum IL-6 levels and serum IgG levels ($r = .74$; $P < .001$).

As well as often having abnormally elevated serum Ig levels, HIV-infected individuals have been reported to frequently have abnormally elevated serum levels of sIL-2R.[33] This is believed to reflect an underlying state of T-cell activation during HIV infection. However, unlike Ig, in vitro studies have demonstrated that IL-6 does not promote, either alone or with mitogenic costimuli, IL-2R expression in T cells.[11,12] We have examined (Fig 6) the relationship between the two parameters (serum IL-6 levels and sIL-2R levels) in 19 HIV-seropositive individuals (stages ½) and found no direct relationship ($r = .01$; $P > .95$). Thus, IL-6, known to induce Ig production in vitro,[10] was generally found to be abnormally elevated in sera of those HIV-infected individuals with high Ig levels. In contrast, IL-6, a T-cell costimulant that does not promote IL-2 secretion or IL-2 receptor expression in human T cells, was found to be present at variable concentrations in sera of HIV-seropositive individuals with high serum levels of sIL-2R.

*Correlation between serum IL-6 levels and isolation of monocytotropic HIV in HIV-seropositive individuals.* HIV preferentially infects CD4 positive lymphocytes and cells of the monocyte/macrophage lineage.[35-37] Because monocytes, but not T cells, are capable of high-level IL-6 secretion,[32] we tested whether increased serum IL-6 levels in HIV-seropositive individuals correlated directly with isolation of monocytotropic virus from these individuals. To this end,

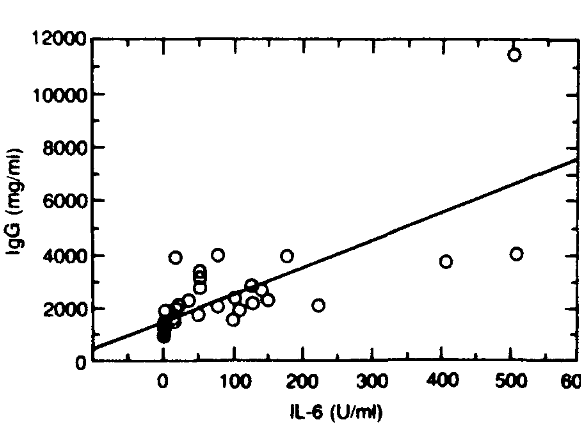


**Fig 5. Serum IgG levels correlate with serum IL-6 in HIV infection. Serum IgG levels were plotted against serum IL-6 levels and correlation calculated by regression analysis. ($r = .74$, $P < .001$).**

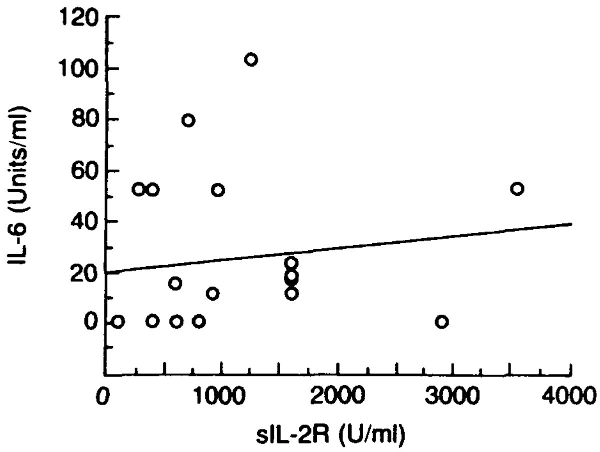


**Fig 6. Relationship between serum levels of sIL-2R and serum IL-6 levels in early-stage HIV infection. Serum levels of sIL-2R were plotted against serum levels of IL-6 and correlation was calculated by regression analysis ($r = .01$, $P > .950$).**

PBMCs from 96 HIV-seropositive individuals (stages 1, 2, and 3) were cocultured both with normal human peripheral blood monocytes and with normal PBMCs. These PBMCs were prestimulated for 3 to 4 days with PHA and IL-2, and thus contained predominantly activated T cells.

In most cases (no. 52) HIV could be isolated from HIV seropositive bloods in both coculture systems. However, these were 24 HIV seropositive blood samples from which virus could only be isolated in coculture with PHA and IL-2 costimulated normal PBMCs, suggesting infection with a predominantly T-cell tropic virus. In addition, there were six HIV-seropositive blood samples from which virus could only be isolated in coculture with normal monocytes, and 14 from which virus could not be isolated in either coculture system.

Mean serum IL-6 levels in 12 randomly chosen HIV-infected individuals who tested virus-positive in PHA-activated PBMC but not in monocyte cocultures were 5.3 U/mL ($\times/\div 1.5$) (Fig 7). In parallel determinations, mean serum IL-6 levels in 12 randomly chosen HIV-infected individuals who tested virus positive in monocyte cocultures were 39 U/mL ($\times/\div 1.9$) ($P < .004$). Together, these findings suggest a correlation between elevated serum IL-6 levels in HIV-seropositive individuals and infection with a monocytotropic strain of HIV.

## DISCUSSION

In this study, we present evidence that a laboratory strain of HIV, HTLV-III$_{Ba\text{-}L}$, as well as natural isolates of HIV from 12 seropositive individuals, promote IL-6 production in culture. Several features characterize in vitro IL-6 production induced by HIV, including a delayed onset after exposure to the virus, the associated occurrence of HIV replication, and the monocyte derivation. The laboratory strain HTLV-III$_{Ba\text{-}L}$ induced IL-6 production in monocyte cultures not before day 11 after addition to culture. At this time the monocytes had began replicating and secreting HIV, as documented by the levels of HIV-associated p24 antigen in culture supernatants. No IL-6 was detected when

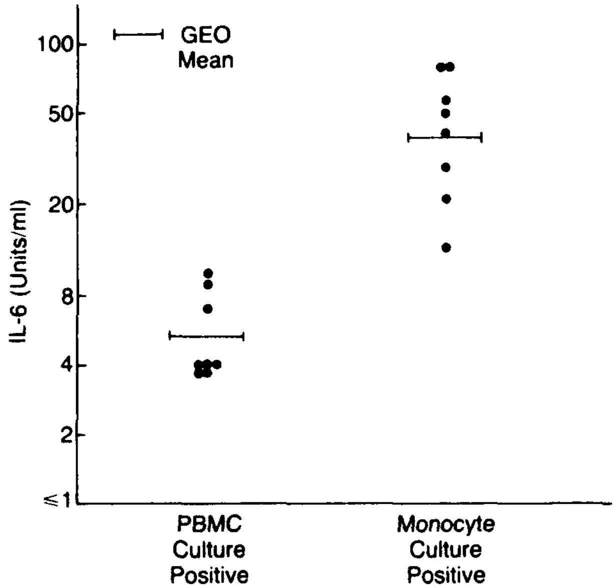


Fig 7. Relationship between infection with monocytotropic HIV and serum IL-6 levels. Serum IL-6 levels were measured in two groups of 12 HIV-seropositive individuals (stages 1, 2, and 3) infected either with HIV capable of replicating in PHA and IL-2 stimulated PBMC (but not in purified monocyte cultures), or with HIV capable of replicating in monocyte cultures.

the virus was inactivated before the addition to monocyte cultures. Similarly, when HIV was derived from virus-infected individuals, secretion of IL-6 in culture occurred after a mean of 36 days of incubation. Secretion of IL-6 in these cultures correlated directly with evidence of HIV production in the same cultures, suggesting that HIV, isolated from HIV-infected blood, was responsible for eliciting IL-6 production.

The experiments presented here indicate that the monocytes are the cellular source of IL-6 production induced by HIV. In one set of experiments, the monocytes represented greater than 95% of the cell type in culture, and thus are the likely source of IL-6 induced by HTLV-III$_{Ba\text{-}L}$. In other experiments that used naturally occurring HIV as the IL-6 inducing agent, the monocytes represented only 50% of the cells in culture. However, among blood cells the monocytes are the principal source of IL-6 production in vitro.[30-32] In addition, the monocytes are the predominant, if not the only, blood cell capable of long-term survival in vitro under the culture conditions used that excluded known T- and B-cell growth factors.

The mechanisms of IL-6 induction by HIV are unclear at present. The experiments presented here show that IL-6 secretion in culture was associated with evidence of HIV production in the same culture. It is possible that the monocytes secreting IL-6 are also those productively infected with HIV. This possibility is supported by the observation that individuals infected with HIV capable of replicating in monocyte cultures had significantly higher serum IL-6 levels than individuals infected with HIV capable of replicating in T-cell, but not monocyte, cultures. However, previous experiments have suggested that HIV can induce a rapid and transient increase of IL-6 gene expression in monocytes without infecting the cells, probably by signal transduction via a cell surface protein.[25] Although the present experiments do not examine these early and transient cell-virus interactions, it could be that HIV, secreted in culture by productively infected monocytes, goes to stimulate IL-6 production in monocytes not infected with the virus. While further studies are required to address these issues, the present experiments demonstrate that HIV replication in monocyte cultures is accompanied by a sustained secretion of IL-6.

If these in vitro events have an in vivo counterpart, one would expect to find elevated levels of IL-6 in HIV-infected individuals. We have found that a proportion of HIV-seropositive individuals with stage 1 through 4 disease have abnormally elevated serum IL-6 levels. In contrast, HIV seropositive patients with advanced disease, stages 5 and 6, displayed serum IL-6 levels not different from normal. Similar findings have recently been reported by others.[38]

Patients with advanced HIV disease are also those who have the greatest HIV burden,[39] and thus may be expected to display the highest levels of serum IL-6. In contrast, we found that patients with advanced HIV disease generally have normal serum IL-6 levels. It is possible that the cells capable of producing IL-6 in vivo are damaged in late-stage HIV disease, and functionally impaired. It is also possible that IL-6 is produced at a high rate in stage 5/6 HIV disease but is mostly bound to carrier proteins that would prevent its detection in biologic assays such as that used here. $\alpha$-2-Microglobulin, identified as a carrier protein for IL-6,[40] has been shown to inhibit the function of a number of growth factors by preventing their binding to the appropriate receptors.[41-43] It is worth noting that serum $\beta$-2-microglobulin levels are markedly elevated in late-stage HIV disease.[44] Finally, it is possible that HIV is not the only, and perhaps, not even the principal inducer of IL-6 production in HIV seropositive individuals. It could be that HIV requires a costimulus (or costimuli) for IL-6 induction that is no longer present in late-stage HIV disease. Further studies are in progress to address these issues.

What might be the role of IL-6 in the pathogenesis of HIV disease? Previous studies have demonstrated that serum IL-6 levels are markedly elevated in severe burn victims, in septic individuals, and in volunteers injected with endotoxin or tumor necrosis factor; IL-6 levels then rapidly return to normal after the acute episode.[18,19,45,46] The role attributed to IL-6 in these conditions is to elicit many of the acute-phase serum protein alterations that usually accompany an acute-phase response. Indeed, IL-6 in vitro is a potent inducer of C-reactive protein, fibrinogen, $\alpha$-1-antichymotrypsin, and $\alpha$-1-antitrypsin in liver cells, and an inhibitor of albumin production in the same cells.[7-9] Abnormally high IL-6 levels have also been reported in sera and synovial fluids of a proportion of patients with rheumatoid arthritis.[21,24] In this illness a significant correlation was found between serum IL-6 levels and disease activity.

Individuals with HIV infection often have circulating activated B cells and abnormally elevated serum Ig levels.[26,47,48]

In addition, a proportion of these individuals display abnormally elevated numbers of EBV-infected B cells in the circulation that might predispose them to the occurrence of EBV-containing B-cell lymphoproliferations.[27] In vitro IL-6 stimulates Ig production in B cells activated by a variety of signals[10,12] and promotes the proliferation of EBV-infected B cells.[2] It is tempting to speculate that IL-6, chronically induced by HIV, and perhaps a variety of other stimuli, are responsible for promoting Ig secretion and expanding the pool of B cells latently infected with EBV in HIV-infected individuals.

An additional possibility is that IL-6 might cause HIV disease progression by promoting T-cell proliferation. In vitro IL-6 stimulates T-cell proliferation[11,12] and it is known that activated T cells replicate HIV in preference to resting T cells.[49,50] Thus, by inducing T-cell proliferation IL-6 may contribute to the expansion of the pool of HIV-infected T cells.

Many questions remain relating to the mechanisms of IL-6 production in HIV disease and the role of IL-6 in the pathogenesis of this illness. However, the results reported here demonstrate that natural isolates of HIV induce IL-6 secretion in vitro and that serum IL-6 levels are abnormally elevated in early-stage HIV disease. These findings provide further evidence for a role of IL-6 as an important mediator of host responses during virus infection.

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## REFERENCES

1. May LT, Ghrayeb J, Santhanam U, Tatter SB, Sthoeger Z, Helfgott DC, Chiorazzi N, Grieninger G, Sehgal PB: Synthesis and secretion of multiple forms of $\beta_2$-interferon/B cell differentiation factor 2/hepatocyte stimulating factor by human fibroblasts and monocytes. J Biol Chem 263:7760, 1988

2. Tosato G, Seamon KB, Goldman ND, Sehgal PB, May LT, Washington GC, Jones KD, Pike SE: Identification of a monocyte-derived human B cell growth factor as interferon-$\beta_2$. Science 239:502, 1988

3. Kirnbauer R, Kock A, Schwarz T, Urbanski A, Krutmann J, Borth W, Damm D, Shipley G, Ansel JC, Luger TA: IFN$\beta_2$-interferon/B cell differentiation factor 2/hybridoma growth factor (IL-6) is expressed and released by human epidermal cells and epidermoid carcinoma cell lines. J Immunol 142:1922, 1989

4. Tabibzadeh SS, Santhanam Y, Sehgal PB, May LT: Cytokine-induced production of IFN-$\beta_2$/IL-6 by freshly explanted human endometrial stromal cells. Modulation by Estradiol-17$\beta$. J Immunol 142:3134, 1989

5. Sehgal PB, Helfgott DC, Santhanam U, Tatter SB, Clarick RH, Ghrayeb J, May LT: Regulation of the acute phase and immune responses in viral disease: Enhanced expression of the "$\beta_2$-interferon/hepatocyte stimulating factor/interleukin-6" gene in virus-infected human fibroblasts. J Exp Med 167:1951, 1988

6. Kohase ML, May T, Tamm I, Vilcek J, Sehgal PB: A cytokine network in human diploid fibroblasts: Interactions of $\beta$ interferons, tumor necrosis factor, platelet-derived growth factor, and interleukin-1. Mol Cell Biol 7:273, 1987

7. Moshage HJ, Roelofs HMJ, van Pelt JF, Hazenberg BPC, van Leeuwen MA, Limburg PC, Aarden LA, Yap SH: The effect of interleukin-1, interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. Biochem Biophys Res Commun 155:112, 1988

8. Ganapathi MK, May LT, Schultz D, Brabenec A, Weinstein J, Sehgal PB, Kushner I: Role of interleukin-6 in regulating synthesis of C-reactive protein and serum amyloid A in human hepatoma cell lines. Biochem Biophys Res Commun 157:271, 1988

9. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H: Interferon $\beta_2$/B-cell stimulatory factor type 2 shares identity with monocyte derived hepatocyte stimulatory factor and regulates the major acute protein response in liver cells. Proc Natl Acad Sci 84:7251, 1987

10. Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, Nakajima K, Pyun KH, Kishimoto T: Purification to homogeneity and characterization of human B cell differentiation factor (BCDF or BSPp-2). Proc Natl Acad Sci 82:5490, 1985

11. Lotz M, Jirik F, Kabouridis P, Tsoukas C, Hirano T, Kishimoto T, Carson DA: B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. J Exp Med 167:1253, 1988

12. Tosato G, Pike S: Interferon $\beta_2$/Interleukin 6 is a costimulant for human T lymphocytes. J Immunol 141:1556, 1988

13. Ikebuchi K, Wong GG, Clark SC: Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 84:9035, 1987

14. Zilberstein A, Ruggieri R, Korn JH, Revel M: Structure and expression of cDNA and genes from human interferon $\beta_2$ a distinct species inducible by growth stimulatory lymphokines. EMBO J 5:2529, 1986

15. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A, Kishimoto T: Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332:83, 1988

16. Klein B, Zhang XG, Jourdan M, Content J, Houssiau F, Aarden L, Piechaczyk M, Bataille R: Paracrine rather than autocrine regulation of myeloma cell growth and differentiation by interleukin-6. Blood 73:517, 1989

17. Revel M, Zilberstein A, Chen L, Gothelf Y, Barash I, Novick D, Rubinstein M, Michalevicz R: Biological activities of recombinant human IFN-$\beta_2$/IL-6 (E. coli). Ann NY Acad Sci 557:144, 1989

18. Nijsten MWN, deGroot ER, tenDuis HJ, Klasen HJ, Hack CE, Aarden LA: Serum levels at 11-6 and acute phase responses. Lancet 2:921, 1987

19. Helfgott DC, Tatter SB, Santhanam U, Clarick RH, Bhardwaj N, May LT, Sehgal PB: Multiple forms of IFN-$\beta_2$/IL-6 in serum and body fluids during acute bacterial infection. J Immunol 142:948, 1989

20. Van Oers MHJ, Van Der Heyden AAPAM, Aarden LA: Interleukin 6 (IL-6) in serum and urine of renal transplant recipients. Clin Exp Immunol 18:1797, 1988

21. Guerne P-A, Zuran BL, Vaughan DA, Carson DA, Lotz M: Synovium as a source of interleukin-6 in vitro. Contribution to local and systemic manifestations of arthritis. J Clin Invest 83:585, 1989

22. Bardwaj N, Santhanam U, Lau LL, Tatter SB, Ghrayeb J, Rivelis H, Steinman RM, Sehgal PB, May LT: Interleukin-6, interleukin-$\beta$2 in synovial effusions of patients with rheumatoid

arthritis and other arthritides: Identification of several isoforms and studies of cellular sources. J Immunol 143:2153, 1989

23. Hirano M, Matsuda T, Turner M, Miyasaka N, Buchan G, Tang B, Sato K, Shimizu M, Maini R, Feldmann M, Kishimoto T: Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 18:1797, 1988

24. Houssiau FA, Devogelaer JP, Van Damme J, deDeuxchaisnes CN, Van Snick J: Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 31:784, 1988

25. Nakajima K, Martinez, Maxa O, Hirano T, Breen EC, Nishanian PG, Salazar-Gonzalez JF, Fahey JL, Kishimoto T: Induction of IL-6 (B cell stimulatory factor 2/IFN/$\beta_2$) production by HIV. J Immunol 142:531, 1989

26. Lane H, Masur H, Edgar LC, Whalew G, Rook AH, Fauci AS: Abnormalities of B cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med 309:453, 1983

27. Birx DL, Redfield RR, Tosato G: Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. N Engl J Med 314:874, 1986

28. Redfield RR, Wright DC, Tramont EC: The Walter Reed staging classification for HTLV-II/LAV infection. N Engl J Med 314:131, 1986

29. Gallo D, Kimpton J, Dailey PJ: Comparative studies on use of fresh and frozen peripheral blood lymphocyte specimens for isolation of human immunodeficiency virus and effects of cell lysis on isolation efficiency. J Clin Microbiol 25:1291, 1987

30. Tosato G, Gerrard TL, Goldman NG, Pike SE: Stimulation of EBV-activated human B cells by monocytes and monocyte products. Role of IFN-$\beta_2$/B cell stimulatory factor 2/11-6. J Immunol 140:4329, 1988

31. Perno C, Yarchoan R, Cooney DA, Hartman NR, Gartner S, Popovic M, Hao Z, Gerrard TL, Wilson YA, Johns DG, Broder S: Inhibition of human immunodeficiency virus (HIV-1/HTLV-$III_{Ba-L}$) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2'-3'-dideoxynucleotides. J Exp Med 118:1111, 1988

32. Aarden LA, DeGroot ER, Schaap DL, Lansdorp PM: Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411, 1987

33. Reddy M, Grieco M: Elevated soluble interleukin-2 receptor levels in serum of human immunodeficiency virus infected populations. AIDS Res Hum Retroviruses 4:115, 1988

34. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

35. Popovic M, Sarngadharan MG, Read E, Gallo RC: Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224:497, 1984

36. Klatzmann D, Barre-Sinoussi F, Nugeyre MT, Dauguet C, Vilmer E, Gricelli C, Brun-Vezinet F, Rouzioux C, Gluckman JC, Chermann J-C, Montagnier L: Selective tropism of lymphadenopathy-associated virus (LAV) for helper-inducer T lymphocytes. Science 225:59, 1984

37. Gartner S, Markovits P, Markovits DM, Kaplan MH, Gallo RC, Popovic M: The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 233:215, 1986

38. Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, Hirano T, Kishimoto T, Martinez-Maza O: Infection with HIV is associated with elevated IL-6 levels and production. J Immunol 144:480, 1990

39. Burke DS, Redfield RR: Transmission of human immunodeficiency virus (HIV). N Engl J Med 318:1202, 1988 (letter)

40. Matsuda T, Hirano T, Nagasawa S, Kishimoto T: Identification of $\alpha$2-macroglobulin as a carrier protein for IL-6. J Immunol 142:148, 1989

41. Raines EW, Daniel FBP, Russell R: Plasma binding proteins for platelet-derived growth factor that inhibit its binding to cell-surface receptors. Proc Natl Acad Sci USA 81:3424, 1984

42. O'Connor-McCourt M, Wakefield LM: Latent transforming growth factor-$\beta$ in serum. J Biol Chem 262:14090, 1987

43. Ronne H, Anunde H, Rask L, Peterson PA: Nerve growth factor binds to serum alpha-2-macroglobulin. Biochem Biophys Res Commun 87:330, 1979

44. Moss AR, Bacchetti P, Osmond D, Krampf W, Chaisson RE, Stites D, Wilber J, Allain JP, Carlson J: Seropositivity for HIV and the development of AIDS or AIDS-related condition: Three year follow-up of the San Francisco General Hospital cohort. Br Med J 296:745, 1988

45. Fong Y, Moldawer LL, Marano M, Wei H, Tatter SB, Clarick RH, Santhanam U, Sherris D, May LT, Sehgal PB, Lowry SF: Endotoxemia elicits increased circulating $\beta_2$-IFN/IL-6 in man. J Immunol 142:2321, 1989

46. Jablongs DM, Mule JJ, McIntosh K, Sehgal PB, May LT, Huang CM, Rosenberg SA, Lotze MT: IL-6/IFN-$\beta_2$ as a circulating hormone. Induction by cytokine administration in humans. J Immunol 142:1542, 1989

47. Yarchoan R, Redfield RR, Broder S: Mechanisms of B cell activation in patients with acquired immunodeficiency syndrome and related disorders. Contribution of antibody-producing B cells, of Epstein-Barr virus-infected B cells, and of immunoglobulin production induced by human T cell lymphotropic virus type III/lymphadenopathy-associated virus. J Clin Invest 78:439, 1986

48. Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajima K, Kishimoto T: The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med 167:332, 1988

49. Hoxie JA, Haggarty BS, Rackowski JL, Pillsbury N, Levy JA: Persistent noncytopathic infection of normal human T lymphocytes with AIDS-associated retrovirus. Science 229:1400, 1985

50. Folks T, Powell DM, Lightfoote MM, Benn S, Martin MA, Fauci S: Induction of HTLV-III/LAV from a non-virus producing T-cell line: Implications for latency. Science 231:600, 1986